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Sp1 and Sp3 Physically Interact and Co-operate with GABP for the Activation of the Utrophin Promoter

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ABSTRACT

The utrophin gene codes for a large cytoskeletal protein closely related to dystrophin which, in the absence of dystrophin, can functionally substitute it. Utrophin is transcribed by two independently regulated promoters about 50 kb apart. The upstream promoter is TATA-less and contains a functional GABP binding site which, in muscle, restricts the promoter activity to post-synaptic nuclei. Transient transfections analysis of mutant promoters in rhabdomyosarcoma cells showed that the upstream promoter contains three functional GC elements that are recognized by Sp1 and Sp3 factors in vitro. Co-transfections of the promoter with Sp1, Sp3 and GABP factors in Drosophila SL2 Schneider cells, which lack of endogenous Sp factors, demonstrated that both Sp1 and Sp3 are positive regulators of the utrophin promoter and that they activate transcription synergistically with GABP. Consistent with this result, we observed physical interaction of both Sp factors with the GABPa subunit in vitro. Functional domain interaction analysis of Sp1 and Sp3 revealed that both factors interact with GABPa through their DNA binding zinc finger domain. The modulation and correct interaction between Sp1, Sp3 and GABP in muscle cells may be critical for the regulation of the utrophin promoter, and provide new targets for therapies of Duchenne muscular dystrophy.

Keywords: utrophin; Sp1; GABP; muscle-transcription; protein-protein

Introduction

The utrophin gene (also named dystrophinrelated gene) is an autosomal homologue of dystrophin (Love et al., 1989), which when mutated is responsible for Duchenne and Becker muscular dystrophies (DMD and BMD, respectively). Utrophin is transcribed in a large mRNA of 13 kb coding for a 395 kDa protein, with up to 73% of amino acid identity with dystrophin in important functional domains (Grady et al., 1997; Pearce et al., 1993). Functional substitution of utrophin with dystrophin in mice has demonstrated that a cure of DMD and BMD up-regulating the utrophin gene in patients is conceivable (Campbell & Crosbie, 1996; Deconinck et al., 1997; Grady et al., 1997; Rafael et al., 1998; Tinsley et al., 1996, 1998). Utrophin isexpressed ubiquitously, although in adult skeletal muscle its expression is mainly restricted to neuromuscular junctions (Gramolini et al., 1997; Khurana et al., 1990; Scho®eld et al., 1993). Utrophin is transcribedby two independently regulated promoters (Burton et al., 1999; Dennis et al., 1996). Theupstream promoter is a TATA-less promoterassociated with a CpG island. It contains severalGC sequences which are putative Sp factor binding sites, and a functional N box (Dennis et al., 1996).This promoter is also under the control of a downstream utrophin enhancer (DUE) localised at about 9 kb within the second intron (Galvagni & Oliviero, 2000). A second promoter is localized about 50 kb further downstream, which gives rise to a utrophin with a different N-terminal domain (Burton et al., 1999). Both promoters drive a wide distribution of utrophin transcripts with overlapping expression in most tissues. The upstream utrophin promoter is mostly expressed in skeletal muscle, while the intronic promoter is more active in heart muscle (Burton et al., 1999; Love et al., 1991).

The upstream utrophin promoter in cultured muscle cells responds by two- to threefold induction to treatment with heregulin or by the transfection of the Ets-related GABP factor (Gramolini et al., 1999; Khurana et al., 1999). GABP has been reported to activate several viral and cellular pro moters (Fromm & Burden, 1998; LaMarco et al., 1991; Thompson et al., 1991; Triezenberg et al., 1988; Virbasius et al., 1993; Watanabe et al., 1993). In response to heregulin, the GABPa protein level is increased and both a and b subunits are phosphorylated (Schaeffer et al., 1998). GABP activates transcription synergistically with several factors, including Sp1, and has been demonstrated to interact directly with ATF and HCF factors (Ding et al., 1999; Dittmer et al., 1994; GeÂgonne et al., 1993; Rosmarin et al., 1998; Sawada et al., 1999; Vogel & Kristie, 2000).

The Sp family of transcription factors is composed of four zinc finger proteins Sp1-4, which in addition to the conserved DNA-binding domain, contain a glutamine-rich activation domain at the N-terminal region. Sp1, Sp3 and Sp4 recognise the consensus GC box element with identical af®nity (Hagen et al., 1992, 1994). Sp4 expression is most abundant in neuronal tissues (Supp et al., 1996), while Sp1 and Sp3 are both ubiquitously expressed (Dynan & Tjian, 1983a,b; Hagen et al., 1994; Kingsley & Winoto, 1992). Sp1-de®cient embryos die after day 10 of embryonic development, while Sp3-deficient mice die at birth as a result of respiratory failure (Bouwman et al., 2000; Marin et al., 1997). Knockout mice phenotypes suggest that both factors have functional redundancy during early embryo development but exert distinct functions at later developmental stages. Sp1 binding to G á C-rich sequences are found in close proximity of transcriptional start sites and in enhancers (Pugh & Tjian, 1990). Accordingly, Sp1 has been shown to be associated both with general coactivators and with several promoter-speci®c transcription activators (Hoey et al., 1993; Kardassis et al., 1999; Lee et al., 1993; Rotheneder et al., 1999; Ryu et al., 1999; Seto et al., 1993). Sp3 has been shown to activate several promoters. It also seems to act as a repressor, since it also contains an inhibitory domain (Hagen et al., 1994; Liang et al., 1996; Majello et al., 1997; Udvadia et al., 1995; Zhao & Chang, 1997). Here, report the identi®cation and characterisation of functional GC sites present on the upstream utrophin promoter. Using in vitro binding experiments and transient transfections, we demonstrated that both Sp1 and Sp3 act as activators and co-operate with GABP to activate the utrophin promoter. We propose that the synergistic transcriptional activation observed is due to direct physical interaction of GABP with both Sp1 and Sp3, and mapped it to the a-subunit of GABP and the DNA-binding zinc ®nger domain of both Sp1 and Sp3 factors.

Results

Sp1 and Sp3 bind to the utrophin promoter The utrophin promoter is a TATA-less promoter rich in GC elements (Dennis et al., 1996). To identify which GC boxes are functionally relevant for the promoter activity, we ®rst performed a DNA footprinting assay with recombinant Sp1 using DNA fragments spaning the promoter region from ÿ352/ á 47 as probes. Sp1 protection from DNase I revealed three main protected sites identi®ed as S1 (ÿ73/ ÿ 27), S2 (ÿ114/ ÿ 96) and S3 (ÿ151/ ÿ 135) (Figures 1 and 2). Interestingly, not all the putative Sp1 sites on the promoter are recognised by Sp1 in vitro. The proximal S1 GC element is a tandem repeat of three non-canonical GC boxes, while the distal S2 and S3 elements are each composed of partially overlapping Sp1 consensus sites (Figure 2). To analyse nuclear factors binding to these elements on the utrophin promoter, we labelled the three probes containing the protected Sp1 sites and performed electrophoretic mobility shift assays (EMSA) with nuclear extracts from rhabdomyosarcoma RD cells. All probes formed similar complexes that were named C1-4 (Figure 3). In addition to the complexes common to all three probes, the DNA fragment S1 showed the formation of the slower migrating complex C5. Retarded complexes were speci®cally competed with an excess of unlabelled homologous oligonucleotides, while non-speci®c oligonucleotides did not affect the formation of the retarded bands (Figure 3(a)). All complexes were strongly reduced and supershifted with anti-Sp1 or anti-Sp3 antibodies (Figure 3(b)). Speci®cally, complexes C1-3 and C5 were inhibited and supershifted with anti-Sp3 antibodies, while the complex C4 was inhibited and supershifted with antibodies anti-Sp1. The presence of multiple bands containing Sp3 is not surprising, since Sp3 has been described to be expressed in at least three variants with different molecular sizes in several tissues (Kennett et al., 1997). The slower migrating band C5 that is formed with probe S1 (Figure 3(a) and (b), lanes 1) must contain more that one Sp3 factor, since this probe contains three protected tandem GC boxes (Figures 1 and 2). Retarded bands with slower mobility than C5 on the S1 probe, which could be observed with longer exposure, were reduced with both anti-Sp1 and anti-Sp3 antibodies (data not shown), suggesting the formation of higher molecular mass complexes. Sp1 and Sp3 activate the utrophin promoter while the distal S2 and S3 elements are each composed of partially overlapping Sp1 consensus sites (Figure 2). To analyse nuclear factors binding to these elements on the utrophin promoter, we labelled the three probes containing the protected Sp1 sites and performed electrophoretic mobility shift assays (EMSA) with nuclear extracts from rhabdomyosarcoma RD cells. All probes formed similar complexes that were named C1-4 (Figure 3). In addition to the complexes common to all three probes, the DNA fragment S1 showed the formation of the slower migrating complex C5. Retarded complexes were speci®cally competed with an excess of unlabelled homologous oligonucleotides, while non-speci®c oligonucleotides did not affect the formation of the retarded bands (Figure 3(a)). All complexes were strongly reduced and supershifted with anti-Sp1 or anti-Sp3 antibodies (Figure 3(b)). Speci®cally, complexes C1-3 and C5 were inhibited and supershifted with anti-Sp3 antibodies, while the complex C4 was inhibited and supershifted with antibodies anti-Sp1. The presence of multiple bands containing Sp3 is not surprising, since Sp3 has been described to be expressed in at least three variants with different molecular sizes in several tissues (Kennett et al., 1997). The slower migrating band C5 that is formed with probe S1 (Figure 3(a) and (b), lanes 1) must contain more that one Sp3 factor, since this probe contains three protected tandem GC boxes (Figures 1 and 2). Retarded bands with slower mobility than C5 on the S1 probe, which could be observed with longer exposure, were reduced with both anti-Sp1 and anti-Sp3 antibodies (data not shown), suggesting the formation of higher molecular mass complexes.

Sp1 and Sp3 activate the utrophin promoter binding to GC boxes

To test the functional role of GC elements, we generated mutants corresponding to the S1, S2 or S3 sites. Wild-type and mutant utrophin promoters in front of the CAT reporter gene were transfected into RD cells. As shown in Figure 4, the deletion of the proximal S1 site affected utrophin promoter activity by over 60 %, while mutations of either S2 or S3 sites affected utrophin promoter function in RD muscle cells by 30 and 20 %, respectively. Direct activation mediated by Sp1 and Sp3 on the utrophin promoter was tested in Drosophila Schneider SL2 cells as these cells lack Sp-like activity. We therefore transfected the wild-type and mutant utrophin-CAT constructs in these cells along with a Drosophila expression vector carrying either the Sp1 ($pP_{ac}Sp1$) or Sp3 ($pP_{ac}USp3$) cDNA. Co-transfection of $pP_{ac}Sp1$ with the utrophin wildtype promoter enhanced the promoter activity in

SL2 cells ca 25-fold (Figure 5). Thus, Sp1 factor is ufficient to activate the utrophin promoter in SL2 cells. By transfecting the promoter carrying the deletion of the proximal S1 binding site we observed a strong reduction of the utrophin promoter activity, while mutation of S2 affected the promoter activity in SL2 cells, but to a lesser extent. Co-transfection of the wild-type and mutant promoters together with Sp3 expression vector demonstrated that this factor is also an activator, albeit at weaker activity when compared with Sp1, since in the same conditions, Sp3 was activating the utrophin promoter of about five- to sixfold (Figure 5).

Sp1 and Sp3 activate the utrophin promoter in co-operation with GABP

The previous experiment demonstrated that both Sp1 and Sp3 activate the utrophin transcription in the heterologous SL2 cells. However, it was previously shown that Sp3 can act either as an activator or as a repressor in different promoter settings (Fandos et al., 1999; Hagen et al., 1994; Kennett et al., 1997; Liang et al., 1996; Majello et al., 1997; Udvadia et al., 1995; Zhao & Chang, 1997). In order to test the activity of these factors in muscle cells we transfected either Sp1 or Sp3 under the control of the CMV promoter in RD cells. Although in these cells we could not observe high levels of activation due to the presence of endogenous factors, by transfecting increasing amounts of either Sp1 or Sp3 expression vector we observed a low, but reproducible, increase of utrophin transcription with both factors (Figure 6). These results allow us to exclude the possibility that the utrophin promoter Sp3 acts as a negative regulator of Sp1 activation.
It has been previously shown that the utrophin promoter contains a functional N box recognised by GABP (Gramolini et al., 1999; Khurana et al., 1999). As Sp1 and GABP have been shown to cooperate in the transcriptional activation of several promoters and enhancers (GeÂgonne et al., 1993; Nuchprayon et al., 1999; Rosmarin et al., 1998), we analysed the the effect of Sp1 and Sp3 activation on a utrophin promoter mutant that has been mutated in the GABP binding site.

Discussion

The upstream utrophin promoter is a typical TATA-less promoter rich in GC residues. It contains a functional N box, recognised by GABP, which has been demonstrated to confer a promoter response to heregulin (Gramolini et al., 1999; Khurana et al., 1999). We now report the identification and characterisation of three functionally distinct GC elements on the promoter: a proximal element composed of three tandem repeated GC boxes, which behaves as a basal promoter element, and two upstream GC boxes required for full promoter activation. All three GC elements of the utrophin promoter are recognised by the ubiquitous Sp1 and Sp3 factors. Sp1 was originally defined as a proximal promoter factor, required for basal promoter activity, which was thought to function only when located in close vicinity from the transcription start site. However, early experiments also showed that Sp1 can function from distant sites as a weak activator (Courey et al., 1989).
In line with these observations, the utrophin GC elements, recognised by Sp1 as well as Sp3 factors, was shown to be necessary for utrophin promoter activity. Although a clear functional distinction between these elements cannot be made, the mutation of the proximal GC tandem repeats strongly impaired the promoter basal activity both in rhabdomyosarcoma and in Sp1/Sp3 transfected Drosophila SL2 cells, while mutations in the distal sites were less effective in the same conditions. Thus, the function of the proximal GC boxes cannot be entirely replaced by the upstream elements in spite of the fact that all three GC elements are extended footprint, and gel retardation experiments revealed the formation of slower migrating complexes that are supershifted with anti-Sp1/Sp3 antibodies. Knockout experiments showed distinct functions of Sp1 and Sp3 during development (Bouwman et al., 2000; Marin et al., 1997). This could be due to the fact that Sp3 can act as an activator or a repressor in different promoter contexts (Fandos et al., 1999; Hagen et al., 1994; Liang et al., 1996; Majello et al., 1997; Udvadia et al., 1995; Zhao & Chang, 1997). On the utrophin promoter, transient transfection experiments revealed that both Sp1 and Sp3 behaved as activators, with Sp1 being more active than Sp3 in both Drosophila and muscle RD cells.
It has been previously demonstrated that GABP activates the utrophin promoter (Gramolini et al., 1999; Khurana et al., 1999). Here, we demonstrated

that GABP synergises with both Sp1 and Sp3 for utrophin promoter activation, and that both Sp1 and Sp3 directly interact with GABP in vitro and in vivo. Mapping the Sp1 and Sp3 interaction domains revealed that both Sp factors interact via their zinc finger DNA-binding domain.

The GABP factor is composed of one Ets-related GABPa subunit and an ankyrin repeat-containing GABPb subunit (Batchelor et al., 1998; LaMarco et al., 1991; Thompson et al., 1991; Watanabe et al., 1993). GST pull-down experiments revealed that only the GABPa subunit interacts with both Sp1 and Sp3 factors. This result was confirmed by immunoprecipitation experiments in which GABPa co-immunoprecipitated with either Sp1 or Sp3.

Thus, our experiments suggest that the synergy between GABP and Sp1/Sp3 is due to direct interaction between the zinc ®nger domain of either Sp1 or Sp3 and the GABPa subunit. Although we observe direct interaction only with GABPa, in order to activate transcription synergistically GABPa/b subunits are required. This result is in line with the observations that the b-subunit is necessary both for DNA binding of the a-subunit and for transactivation of the heteromeric GABP factor (Batchelor et al., 1998; Guneja et al., 1995, 1996; Thompson et al., 1991). Thus, the direct interaction between Sp1/Sp3 and GABPa subunit is able to recruit to the utrophin promoter the active GABPa/b complex. Our experiments also suggest that post-translational modifications are not required for interaction between GABP and Sp1/Sp3, since we observed direct binding of these factors synthesised in vitro. Interestingly, it has been previously demonstrated that stimulation of muscle cells with heregulin increases GABPa protein levels besi de inducing the phosphorylation of both a and b-subunits (Altiok et al., 1997; Fromm & Burden, 1998; Schaeffer et al., 1998). Moreover, using transient transfection experiments it was also demonstrated that the co-transfection of GABP cDNAs, along with the utrophin promoter in muscle cells, resulted in the activation of the promoter transcription to levels comparable to those obtained with cell treatment with heregulin (Gramolini et al., 1999; Khurana et al., 1999).

Thus, the increase of GABP per se is sufficient to induce utrophin up-regulation, suggesting that GABPa is present in limiting amounts in muscle cells. Small increase of GABPa can, when interacting with GABPb, synergise with either Sp1 or Sp3 and increase utrophin transcription.

In addition, in post-synaptic nuclei it has been shown that Sp1 is phosphorylated in response to synaptic-speci®c stimuli suggesting that, upon phosphorylation, Sp1 may increase its binding to DNA via protein-protein interactions, facilitating the formation of Sp1 multimers and/or increasing its interaction with other proteins (Alroy et al., 1999). Thus, it is possible that Sp1 phosphorylation may allow a fine modulation of the utrophin promoter in response to extracellular stimuli. Further studies will address the role of Sp and GABP phosphorylations with respect to their interaction. This could be relevant in view of the possible regulation of the utrophin transcription by pharmacological means in DMD and BMD patients.